LIPOSOME COMPOSITIONS AND METHODS FOR THE TREATMENT OF ATHEROSCLEROSIS

BACKGROUND OF THE INVENTION

The present invention provides pharmaceutical compositions and methods useful for the treatment of atherosclerosis. More particularly, the compositions generally comprise liposomes having an average diameter of about 100-150 nanometers and a pharmaceutically acceptable carrier. The methods generally comprise administering such compositions.

Atherosclerosis is the leading cause of death in the United States. Atherosclerosis is the formation of plaques in arterial walls that can occlude the vessel lumen and obstruct blood flow through the vessel. Morbidity and mortality generally occur through end organ damage and organ dysfunction resulting from ischemia. The most common forms of ischemic end organ damage are myocardial infarction and cerebrovascular accidents. Disability or death often result from these vascular events. Even atherosclerosis-related ischemia that does not permanently injure myocardium is responsible for significant morbidity in the form of angina pectoris and congestive heart failure. Other organs, such as the kidneys, the intestines, and the spinal cord, may also be injured by atherosclerotic occlusions. Further, in diseases such as aortic aneurysms, atherosclerotic arteries may cause clinical symptoms independent of end organ dysfunction.

Arteriosclerotic lesions are plaques that form by accumulation of cholesterol, cholesterol esters, and phospholipids and proliferation of smooth muscle cells in the intima of major arteries. Lipid contributes a major portion of the plaque volume (generally 30-65% dry weight). Small, Arteriosclerosis, 8:103-129 (1988). In fact, the risk of

developing arteriosclerosis is directly related to the concentration of certain forms of plasma cholesterol.

Lipids, including cholesterol, are generally insoluble in aqueous plasma. Plasma lipids are carried by soluble lipoprotein complexes. These lipoprotein complexes consist of an inner core of non-polar lipids (cholesteryl esters and triglycerides) and an surface layer of hydrophilic proteins and polar lipids (phospholipids and non-esterified cholesterol). Different proteins are present in the surface coat of different lipoprotein complexes (lipoproteins). The different lipoproteins perform different functions in lipid metabolism.

Five classes of lipoproteins are known. Some lipoproteins carry triglycerides and cholesterol from the liver to peripheral tissues while others transport lipids to the liver. Cholesterol may be metabolized in the liver to bile salts that are excreted, thus lowering total body cholesterol. Two lipoproteins, low density lipoproteins (LDL) and high density lipoproteins (HDL), have a high degree of association with the development of atherosclerosis. LDL has a high cholesterol concentration, delivers lipids to cells of peripheral tissues, and is associated with a high risk of atherosclerosis. HDL also has a relatively high cholesterol concentration, but carries lipids to the liver for metabolism into bile salts and is associated with decreasing the risk of developing atherosclerosis.

Cholesterol metabolism and homeostasis is the result of a complex equilibrium between free sterol in the cell and in plasma. Phillips et al., <u>Biochim. Biophys. Acta</u>, 906:223-276 (1987). Delivery of cholesterol to cells occurs via the receptor-mediated LDL pathway and by passive exchange of sterol between plasma membranes and lipoproteins. Only tissues that produce steroid hormones and bile acids can metabolize cholesterol. In order to prevent accumulation of excess free sterol in remaining peripheral tissues there is a reverse transport of cholesterol from plasma membranes into HDL and lipoprotein-like particles. HDL transports excess cholesterol to the liver where it can either be processed into

bile salts for excretion or incorporated into very low density lipoproteins (VLDL) to re-enter the lipoprotein pool.

The passive exchange of cholesterol between cells and lipoproteins occurs via the diffusion of sterol molecules across the aqueous space. Phillips et al., supra, and Schroeder et al., Exp. Biol. Med., 196:235-252 (1991). Net cellular efflux occurs if the chemical potential of free cholesterol is lower in the plasma than in the cells so that sterol leaves the membrane following its activity gradient. Under these conditions, it has been shown that cholesterol-ester-loaded cells, which are morphologically characteristic of early atherosclerotic lesions, not only lose cholesterol, but promote ester hydrolysis, resulting in the reduction of intracellular deposits of this lipid. Small, Arteriosclerosis, 8:103-129 (1988). Moreover as mentioned above, there is epidemiological evidence that conditions which might be expected to enhance reverse cholesterol transport (low plasma cholesterol concentrations, or increased HDL concentrations) are correlated with reduced risk of premature atherosclerosis and may give rise to plaque regression.

Characteristically, plaques are associated with ulceration of the vessel intima. The lipid-containing plaques grow in the ulcerations projecting friable masses into the arterial lumen. The plaques may also injure and weaken the smooth muscle media of the vessel. As plaque formation progresses, more central regions of the plaques are shielded from the circulation. Extensive plaque formation also cause concentric constriction of the vessel at the plaque site.

Presently, the most effective treatment of atherosclerosis is prevention. There is evidence that the progression and accumulation of lipids in lesions can be halted when plasma LDL concentrations are kept to near normal levels. Reynolds, Circulation, 79:1146-1148 (1989). Current preventive management of atherosclerotic disease has focused on the use of drugs in conjunction with dietary restrictions to regulate plasma cholesterol levels. Moreover, antioxidant therapies which suppress the formation and uptake of modified LDL particles by the cells of the arterial wall are also

proving beneficial. Chisolm, <u>Clin. Cardiol.</u>, 14:25-30 (1991).
However, while hypocholesterolemic drugs induce favorable
plasma cholesterol changes which appear to slow the
progression of atherosclerosis, they do not generally induce
conditions that promote the efflux and removal of atheroma
cholesterol. Clearly, in order to achieve significant
regression of atheroma and lessen lumen obstruction, these
space occupying lipids must be mobilized. Present evidence
suggests that processes which stimulate the efflux of
extrahepatic cell cholesterol and transport it to the liver
for excretion, reverse cholesterol transport (RCT), are
important events in the prevention of atherosclerosis.
Gwynne, <u>Clin. Cardiol.</u>, 14:17-24 (1991).

Current therapeutic modalities of arteriosclerosis
are generally divided into surgical and medical management.
Surgical therapy may entail vascular graft procedures to
bypass regions of occlusion (e.g., coronary artery bypass
grafting), removal of occluding plaques from the arterial wall
(e.g., carotid endarterectomy), or percutaneously cracking the
plaques (e.g., balloon angioplasty). Surgical therapies carry
significant risk and only treat isolated lesions.
Atherosclerotic plaques downstream from the treated lesion may
continue to obstruct blood flow. Surgical therapies also do
not limit the progression of atherosclerosis and are
associated with the late complication of restenosis.

Medical therapy is directed to reducing other risk
factors related to vascular disease (e.g., smoking, diabetes,
and hypertension) and lowering forms of serum cholesterol that
are associated with the development of atherosclerosis as
described above. While medical therapies may slow the
progression of plaque formation, plaque regression is
relatively rare. Therefore, symptomatic atherosclerosis often
requires both surgical and medical treatment.

Paradoxically, intravenous infusion of phospholipids
and liposomes has been shown to produce regression of
atherosclerotic plaques although serum lipid levels are
transiently elevated. Williams et al., <u>Perspect. Biol. Med.</u>,
27:417-431 (1984). In some instances, however, cholesterol

associated with development and progression of atherosclerosis
may increase following liposome administration.

Previous studies investigating phospholipid-induced
mobilization of cholesterol in vivo have employed
5   multilamellar or sonicated liposome vesicles. Liposome size
is a key characteristic in clearance kinetics and is one of
several reasons why sonicated vesicles have been expected to
represent the bilayer structure best suited to enhance reverse
cholesterol transport. Sonication reduces multilamellar
10   vesicles (MLV) to 'limit size' vesicles. These systems
exhibit the minimum radius of curvature that can be adopted by
the bilayer configuration without disruption. For example,
the minimum size egg phosphatidylcholine liposome that can be
generated is typically about 30-nm diameter, often classified
15   as a small unilamellar vesicle (SUV). For a given liposome
composition, it is generally assumed that the smaller the
particle diameter the greater the circulation half-life
(Gregoriadis and Senior, Life Sci., 113:183-192 (1986)).
Consequently, it was expected that SUV composed of
20   phosphatidylcholine would circulate longer than larger
liposomes, and therefore mobilize more cholesterol.
Furthermore, packing constraints experienced by phospholipids
in SUV, (due to the acute radius of curvature) gives rise to
an instability that can result in fusion, Hope et al., Chem.
25   Phys. Lipids, 40:89-107 (1986), as well as an increased
tendency to assimilate with lipoproteins. See, e.g.,
Scherphof et al., Biochim. Biophys. Acta, 542:296-307 (1978)
and Krupp et al., Biochim. Biophys. Acta, 72:1251-1258 (1976).
Therefore, it was expected that SUV would produce a greater
30   number of HDL-like particles, thus promoting efflux of sterol
from peripheral tissues. Supporting this expectation,
liposomes having diameters of 50-80 nm have been reported to
optimize sterol mobilization and plaque regression. European
Patent Publication No. 0461559A2.

35   What is needed in the art is a medical treatment for
atherosclerosis that not only will slow progression of
lesions, but also predictably cause regression and shrinkage
of established plaques. Such a treatment should provide the

optimal rate of cholesterol removal (and, hence shrinkage) from plaques. Quite surprisingly, the present invention fulfills these and other related needs.

SUMMARY OF THE INVENTION

The present invention provides pharmaceutical compositions consisting essentially of unilamellar liposomes having an average diameter of about 100-150 nanometers, which liposomes are not bound to a drug; and a pharmaceutically acceptable carrier. These liposomes optimize cholesterol efflux from atherosclerotic plaques. The liposomes may be bound to an apoprotein, typically apoprotein A1 or A2. The liposomes often contain at least one phospholipid, such as phosphatidylcholine or phosphatidylglycerol. Liposomes having diameters of about 125 nm are preferred.

Also provided are methods for treating atherosclerosis employing the pharmaceutical compositions of the present invention. The compositions are administered to animals having atherosclerosis. Often, the compositions will be serially administered over a period of time. Generally, the compositions will be administered parenterally, usually intravenously. The methods may be employed therapeutically or prophylactically. The methods of the present invention are also useful for treatment of hypoalphalipoproteinemia and hyperlipidemias.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 demonstrates cholesterol mobilization by a homogeneous population of large unilamellar vesicles with a mean diameter of 125 nm.

Fig. 2 illustrates plasma cholesterol distribution in normal and liposome-treated animals.

Fig. 3 illustrates liposome cholesterol accumulation over a 24-h time-course in vivo.

Fig. 4 illustrates cholesterol mobilization by liposomes.

Fig. 5 illustrates a comparison of the rate of cholesterol accumulation by unilamellar and oligolamellar liposomes.

Fig. 6 demonstrates the cholesterol mobilizing ability of liposomes having different compositions.

Fig. 7 illustrates the cholesterol content of erythrocytes in mice treated with liposomes and untreated mice.

Fig. 8 illustrates plasma cholesterol concentration changes in rabbits treated with liposomes and untreated rabbits.

Fig. 9 illustrates plasma phospholipid concentration changes in rabbits treated with liposomes and untreated rabbits.

Fig. 10 demonstrates the quantity of cholesterol mobilized by liposomes during treatment of rabbits.

Fig. 11 illustrates clearance profiles of liposomes injected into rabbits.

Fig. 12 demonstrates the cholesterol:phospholipid ratio of lipoproteins following liposome injection.

Fig. 13 illustrates aortic cholesterol content in liposome and saline treated rabbits.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides pharmaceutical compositions consisting essentially of unilamellar liposomes having an average diameter of about 100-150 nanometers, which liposomes are not bound to a drug; and a pharmaceutically acceptable carrier. Also provided are methods for treating atherosclerosis using the compositions of the present invention.

As used herein, "drug" is meant to indicate a synthetic compound suitable for therapeutic use without associated bound carriers, adjuvants, activators, or co-factors. "Drug" does not include apoproteins, lecithin-cholesterol acyltransferase, or albumin.

"Liposome", "vesicle" and "liposome vesicle" will be understood to indicate structures having lipid-containing

membranes enclosing an aqueous interior. The structures may have or one more lipid membranes unless otherwise indicated, although generally the liposomes will have only one membrane. Such single layered liposomes are referred to herein as

5   "unilamellar".

Arterial atherosclerotic lesions have been shown to regress when treated with liposome infusions. In some instances, however, LDL cholesterol has been observed to increase following liposome administration. Prior to the

10   present invention, the cause of this paradox has not been understood.

The present invention is based, in part, on the discovery that liposome size plays a critical role in the metabolism of cholesterol removed from atherosclerotic plaques

15   by the liposomes. Contrary to previous descriptions of liposome therapy, liposomes having a diameter of greater than 100 nanometers are more effective for removing cholesterol from atherosclerotic plaques than smaller liposomes.

The superior action of liposomes greater than 100

20   nanometers in diameter may be explained by the micro-anatomy of the liver. When circulating in the liver, large liposomes (as used herein, liposomes greater than 100 nm in diameter) may be cleared by the Kupffer cells that line the sinusoidal openings. The Kupffer cells transfer cholesterol to

25   hepatocytes for excretion in the bile or re-utilization. Small liposomes (as used herein, liposomes smaller than 100 nm) may directly access hepatocytes without prior processing by the Kupffer cells. Because these small liposomes are infused in relatively large doses, hepatocytes may be acutely

30   exposed to a relatively high concentration of small liposomes and their accumulated cholesterol.

The pharmaceutical compositions of the present invention generally consist essentially of unilamellar liposomes having an average diameter of about 100-150

35   nanometers, which liposomes are not bound to a drug; and a pharmaceutically acceptable carrier. In some instances multilamellar liposomes may also be employed in the compositions of the present invention, either exclusively or

in addition to unilamellar liposomes. The liposomes will have an average diameter of about 100-150 nanometers, typically about 125-140 nanometers. In some embodiments, liposomes having an average diameter larger than 150 nanometers, either

5   uni- or multilamellar, may also be present in the compositions of the present invention.

Persons of skill will appreciate that the liposomes in the compositions of the present invention may be synthesized by a variety of methods, such as described in,

10   e.g., U.S. Patent No. 4,186,183; U.S. Patent No. 4,217,344; U.S. Patent No. 4,261,975; U.S. Patent No. 4,485,054; U.S. Patent No. 4,774,085; U.S. Patent No. 4,946,787; PCT Publication No. WO 91/17424, Deamer and Bangham, Biochim. Biophys. Acta, 443:629-634 (1976); Fraley et al., Proc. Natl.

15   Acad. Sci. USA, 76:3348-3352 (1979); Hope et al., Biochim. Biophys. Acta, 812:55-65 (1985); Mayer et al., Biochim. Biophys. Acta, 858:161-168 (1986); and Williams et al., Proc. Natl. Acad. Sci., 85:242-246 (1988), each of which is incorporated herein by reference. Suitable methods include,

20   e.g., sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles, and ether-infusion methods, all well known in the art.

Generally, the liposomes are most conveniently

25   generated by sonication and extrusion procedures. Briefly, a chloroform solution of lipid is vortexed and the solvent removed under a steady stream of N_2. The sample is dried under a high vacuum. The resulting dry lipid film is rehydrated in 150 mM NaCl and 20 mM [4-(2-hydroxyethyl)]-

30   piperazine-ethanesulfonic acid (Hepes, pH 7.4). This generally produces multilamellar liposomal vesicles. Unilamellar vesicles are prepared by sonication or extrusion.

Sonication is generally performed with a tip sonifier, such as a Branson tip sonifier, in an ice bath.

35   Typically, the suspension is subjected to several sonication cycles. Extrusion may be carried out by biomembrane extruders, such as the Lipex Biomembrane Extruder. Defined pore size in the extrusion filters may generate unilamellar

liposomal vesicles of specific sizes. The liposomes may also
be formed by extrusion through an asymmetric ceramic filter,
such as a Ceraflow Microfilter, commercially available from
the Norton Company, Worcester MA.

5   The size of the liposomal vesicles may be determined
by quasi-electric light scattering (QELS) as described in
Bloomfield, Ann. Rev. Biophys. Bioeng., 10:421-450 (1981),
incorporated herein by reference. Average liposome diameter
may be reduced by sonication of formed liposomes.
10   Intermittent sonication cycles may be alternated with QELS
assessment to guide efficient liposome synthesis.

The liposomes may be composed of a variety of
lipids. Generally, the liposomes will be composed of at least
one phospholipid, typically egg phosphatidylcholine, egg
15   phosphatidylglycerol, distearoylphosphatidylcholine, or
distearoylphosphatidylglycerol. Many embodiments of the
present invention will include more than one phospholipid.

Other phospholipids suitable for formation of
liposomes comprising the compositions of the present invention
20   include, e.g., phosphatidylcholine, phosphatidylglycerol,
lecithin, β,γ-dipalmitoyl-α-lecithin, sphingomyelin,
phosphatidylserine, phosphatidic acid, N-(2,3-di(9-(Z)-
octadecenyloxy))-prop-1-yl-N,N,N-trimethylammonium chloride,
phosphatidylethanolamine, lysolecithin,
25   lysophosphatidylethanolamine, phosphatidylinositol, cephalin,
cardiolipin, cerebrosides, dicetylphosphate,
dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine,
dipalmitoylphosphatidylglycerol, dioleoylphosphatidylglycerol,
palmitoyl-oleoyl-phosphatidylcholine, di-stearoyl-
30   phosphatidylcholine, stearoyl-palmitoyl-phosphatidylcholine,
di-palmitoyl-phosphatidylethanolamine, di-stearoyl-
phosphatidylethanolamine, di-myrstoyl-phosphatidylserine, di-
oleyl-phosphatidylcholine, and the like. Non-phosphorus
containing lipids may also be used in the liposomes of the
35   compositions of the present invention. These include, e.g.,
stearylamine, docecylamine, acetyl palmitate, fatty acid
amides, and the like. Additional lipids suitable for use in
the liposomes of the present invention are well known to

persons of skill in the art and are cited in a variety of well known sources, e.g., McCutcheon's Detergents and Emulsifiers and McCutcheon's Functional Materials, Allured Publishing Co., Ridgewood, N.J., both of which are incorporated herein by

5   reference.

Generally, it is desirable that the liposomes be composed of lipids that are liquid-crystalline at 37°C, often at 35°C, and even 32°C. Liposomes in the liquid-crystalline state typically accept cholesterol more efficiently than

10 liposomes in the gel state. As patients typically have a core temperature of about 37°C, liposomes composed of lipids that are liquid-crystalline at 37°C are generally in a liquid-crystalline state during treatment and, therefore, optimize removal of cholesterol from plaques.

15 The pharmaceutical compositions of the present invention also comprise a pharmaceutically acceptable carrier. Many pharmaceutically acceptable carriers may be employed in the compositions of the present invention. Generally, normal saline will be employed as the pharmaceutically acceptable

20 carrier. Other suitable carriers include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. These compositions may be sterilized by conventional, well known sterilization

25 techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances

30 as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The concentration of liposomes in the carrier may

35 vary. Generally, the concentration will be about 20-200 mg/ml, usually about 50-150 mg/ml, and most usually about 100 mg/ml. Persons of skill may vary these concentrations to optimize treatment with different liposomal components or of

particular patients. For example, the concentration may be
increased to lower the fluid load associated with treatment.
This may be particularly desirable in patients having
atherosclerosis-associated congestive heart failure or severe

5   hypertension. Alternatively, liposomes composed of irritating
lipids may be diluted to low concentrations to lessen
inflammation at the site of administration.

The liposomes may also be bound to a variety of
proteins and polypeptides to increase the rate of cholesterol

10   transfer or the cholesterol-carrying capacity of the
liposomes. Binding of apoproteins to the liposomes is
particularly useful. As used herein, "bound to liposomes" or
"binding to liposomes" indicates that the subject compound is
covalently or non-covalently bound to the surface of the

15   liposome or contained, wholly or partially, in the interior of
the liposome. Apoprotein A_1, apoprotein A_2, and apoprotein E
will generally be the most useful apoproteins to bind to the
liposomes. These apoproteins promote transfer of cholesterol
and cholesteryl esters to the liver for metabolism. Lecithin-

20   cholesterol acyltransferase is also useful for metabolizing
free cholesterol to cholesteryl esters. Liposomes in the
pharmaceutical compositions of the present invention may be
bound to molecules of apoprotein A_1, apoprotein A_2, and
lecithin-cholesterol acyltransferase, singly or in any

25   combination and molar ratio. Additional proteins or other
non-protein molecules may also be useful to bind to the
liposomes to enhance liposome stability or half-life and the
like. These include, e.g., cholesterol, polyethyleneglycol,
alkylsulfates, ammonium bromide, albumin, and the like.

30   Also provided are methods for treating
atherosclerosis in an animal. The methods generally comprise
administering a liposome composition to the animal, which
liposome composition consists essentially of unilamellar
liposomes having an average diameter of about 100-150

35   nanometers. By "treating atherosclerosis", it is meant
performing a therapeutic intervention that results in reducing
the cholesterol content of at least one atherosclerotic plaque
or prophylactically inhibiting or preventing the formation or

expansion of an atherosclerotic plaque. Generally, the volume of the atherosclerotic plaque, and hence the degree of obstruction of the vascular lumen, will also be reduced. The present methods are particularly useful for treating

5   atherosclerotic lesions associated with familial hyperlipidemias.

The methods of the present invention may reduce the cholesterol content of atherosclerotic plaques and/or the volume of atherosclerotic plaques. The cholesterol content

10   will generally be reduced by at least 10%-30%, often by 30%-50%, and in some instances as much as 75%-85% or more. The volume of the atherosclerotic plaques will also generally be reduced. The reduction in plaque volume will generally be at least 5%-30%, often as much as 50%, and in some instances 75%

15   or more.

Cholesterol may be mobilized from the plaques by either direct efflux into the liposomes or into lipoproteins that subsequently transfer the cholesterol to the liposomes. As cholesterol is transferred to the liposomes from the

20   lipoproteins, the lipoproteins may receive more cholesterol from plaques. Generally, when cholesterol is received from lipoproteins, the cholesterol is transferred from HDL.

The methods may be useful to treat atherosclerosis in a variety of animals and in a variety of blood vessels.

25   Typically, the animal will be human, although non-human primates, dogs, cats, rodents, horses, cows, and the like may be treated by the methods of the present invention. Atherosclerosis of any blood vessel, such as the aorta, carotid arteries (common, internal, and external), coronary

30   arteries, mesenteric arteries, renal arteries, iliac arteries, popliteal arteries, and the like, may also be treated by the methods of the present invention.

The methods may also be useful for prophylactic treatments. Such prophylactic treatments are particularly

35   useful following invasive vascular procedures. Vascular regions having injured endothelium are at increased risk for developing atherosclerotic plaques. Therefore, invasive vascular procedures, such as coronary angioplasty, vascular

bypass grafting, and other procedures that injure the vascular endothelial layer, may be practiced in conjunction with the methods of the present invention. As the invasive procedure injures the endothelium, the liposomes act to remove

5   cholesterol from the injured region and inhibit or prevent plaque formation of expansion during endothelial healing.

Hyperlipidemias may also be treated by the methods of the present invention. Administration of liposomes, alone or bound to apoprotein A_1 and apoprotein A_2, to individuals

10 having hypoalphalipoproteinemia from genetic or secondary causes, familial combined hyperlipidemia, and familial hypercholesterolemia is a useful treatment.

The liposomes administered in the methods of the present invention will be composed of lipids as described

15 above. The lipids will generally be in the liquid-crystalline state at 37 °C. The lipids will also generally include one or more phospholipids, often phosphatidylcholine or phosphatidylglycerol, although liposomes may be composed of many other lipids, examples of which are described above.

20 The liposomes may be administered in many ways. These include parenteral routes of administration, such as intravenous, intramuscular, subcutaneous, and intraarterial. Generally, the liposomes will be administered intravenously. Often, the liposomes will be administered into a large central

25 vein, such as the superior vena cava or inferior vena cava, to allow highly concentrated solutions to be administered into large volume and flow vessels. The liposomes may be administered intraarterially following vascular procedures to deliver a high concentration directly to an affected vessel.

30 The liposomes may also be administered directly to vessels in a topical manner by surgeons during open procedures. In some instances, the liposomes may be administered orally or transdermally. The liposomes may also be incorporated in vascular stents for long duration release following placement.

35 This is particularly effective for angioplasty treatment of restenosis of lesions in the coronary arteries.

As described above, the liposomes will generally be administered intravenously in the methods of the present

invention. Often multiple treatments will be given to the patient, generally weekly. Typically, the therapy will continue for about 4-16 weeks (4-16 treatments), usually about 10 weeks (10 treatments). The duration and schedule of

5   treatments may be varied by methods well known to those of skill.

The dose of liposomes may vary depending on the clinical condition and size of the animal or patient receiving treatment. Humans will generally be treated with about 0.1-

10 1.5 gm of liposomes/kg body weight, usually about 0.2-0.75 gm gm/kg, and most usually about 0.28-0.42 gm/kg. Thus, an average 70 kg person would be treated with about 20-30 gms. of liposomes per treatment. The dose will typically be constant over the course of treatment, although the dose may vary.

15 Serum measurements of total free cholesterol, total esterified cholesterol, HDL cholesterol, LDL cholesterol, and VLDL cholesterol may be used to assess and modify dosage amounts and schedules during the treatment regimen. As cholesterol is mobilized from plaques, total serum cholesterol rises. It is

20 desirable that total serum cholesterol and HDL cholesterol rise during therapy, and esterified cholesterol drop during therapy. The liposome dose for different animals will generally approximate the human weight-determined dosage.

The following examples are offered by way of

25 illustration and not limitation.

30

EXAMPLES

Example 1 -- Influence of Liposome Size and Composition on In Vivo Cholesterol Mobilization

35 This example demonstrates the relative cholesterol mobilizing efficacy of liposomes of different sizes and compositions in mice. Liposomes having a mean diameter of about 125 nm were found to be the most effective in mobilizing

cholesterol in vivo. Liquid-crystalline liposomes were more
effective in mobilizing cholesterol than gel-state liposomes.

     Cholesterol and [4-(2-hydroxyethyl)]-
piperazineethanesulfonic acid (Hepes) were obtained from

5   Sigma. [^{14}C]cholesterol hexadecyl ether and [^{3}H]cholesterol
were purchased from New England Nuclear. Egg
phosphatidylcholine (EPC), distearoylphosphatidylcholine
(DSPC), distearoylphosphatidylglycerol (DSPG) and egg
phosphatidylglycerol (EPG) were supplied by Avanti Polar

10   Lipids. Bio-Gel^{TM} A-15m medium was purchased from Bio-Rad. All
chemicals, thin layer chromatography plates and solvents were
of analytical grade and purchased from BDH Chemicals.

    All liposome preparations were labelled using trace
amounts of [^{14}C]cholesterol hexadecyl ether (CHE). This

15   labelling is useful as (1) it does not undergo passive
exchange between membranes; (2) mice do not exhibit
cholesterol-ester exchange protein activity; and (3) the
ether-linked fatty acid is not cleaved in the plasma.
Consequently, in this model system CHE is an excellent

20   liposome marker and vesicle concentrations in the plasma were
estimated from the specific activity of this label.

    A chloroform solution of EPC and [^{14}C]CHE was
vortexed and solvent was removed under a stream of N_2. The
sample was dried under high vacuum for 2 h. The dry lipid

25   film was hydrated in 150 mM NaCl, 20 mM Hepes (pH 7.4) to
generate multilamellar vesicles (MLV). Vesicles were prepared
from MLV either by sonication, to generate small unilamellar
vesicles (SUV) or extrusion to produce large unilamellar
vesicles (LUV). Sonication was performed using a Branson tip

30   sonifier, following standard protocols. The MLV suspension
was diluted to 30 mg/ml, immersed in an ice bath and subjected
to 3 cycles of sonication, each of 10-min duration. The
initial milky suspension became clear and the vesicle size was
30 nm, as determined by quasi-elastic light scattering (QELS).

35   The SUV were centrifuged at 10000Xg for 30 min to remove
titanium fragments originating from the sonicator tip.

    Extrusion was carried out using a 10 ml Lipex
Biomembranes Extruder equipped with a water jacketed

thermobarrel as described by Hope et al., *Biochim. Biophys. Acta*, 812:55-65 (1985), incorporated herein by reference. MLV were sized through two stacked polycarbonate filters of defined pore size to generate a variety of LUV and homogeneous

5   MLV as described in Hope et al., *supra*, and Mayer et al., *Biochim. Biophys. Acta*, 858:161-168 (1986), incorporated herein by reference.

The size of vesicles generated by sonication and extrusion procedures was determined by QELS analysis utilizing

10   a Nicomp Model 370 submicron laser particle sizer equipped with a 5-mW He-Ne Laser. The Nicomp QELS analyzes fluctuations in light-scattering intensities due to vesicle diffusion in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and thus, the

15   mean diameter of vesicles. The following diameters are expressed as the mean ±S.D. of vesicle preparations prior to injection. Vesicles prepared by sonication were 30±7 nm in diameter (SUV_{30}). Vesicles prepared by extrusion through filters with a pore size of 0.05 μm were 70±19 nm, 0.1 μm pore

20   size were 125±30, and 0.4 μm pore size were 237±90 nm. Generally, the vesicles prepared by extrusion are referred to herein by the filter pore size used in their preparation, i.e., LUV_{50}, LUV_{100} and MLV_{400}.

Female BDF-1 or CD-1 mice, weighing 20-22 g

25   (Sprague-Dawley), were used throughout this study. Liposomes were injected via the tail vein at a dose of 300 mg/kg, which was typically 6 mg of liposomes in 200 ml of buffer injected for each animal. Control mice were injected with an equal volume of buffer and both groups were sacrificed at specified

30   times with blood collection in EDTA microtainer tubes by heart puncture. Plasma was obtained following centrifugation at 2000 x g for 10 min, and an aliquot removed for scintillation analysis using a Beckman LS 3801 liquid scintillation counter. The average of data from 16 mice (from four separate

35   experiments) is indicated at each time point, unless indicated otherwise.

A 27 x 1.5 cm Bio-Gel A-15m gel filtration column, equilibrated with 150 mM NaCl, 10 mM Tris, 0.1% EDTA, 0.3%

NaN_3 (pH 7.4) was used to fractionate plasma samples. Columns were eluted at a flow rate of 1 ml/min and 1-ml fractions were collected for radioactivity and lipid analyses. Data on the cholesterol:phospholipid (C/P) ratio of vesicles and lipoproteins after infusion was obtained from pooled fractions corresponding to the liposomal and lipoprotein peaks. The Bio-Gel columns were calibrated with respect to lipoprotein elution by preparing purified human lipoprotein fractions using standard ultracentrifugation procedures as described in Schumaker et al., Methods Enzymol., 128:155-181 (1986), incorporated herein by reference. The lipoprotein fractions were each labelled with [3H]cholesterol. The elution profiles of the columns were monitored for radioactivity.

Pooled column fractions and plasma samples were extracted employing the Bligh and Dyer procedure. Bligh and Dyer, Can. J. Biochem. Physiol., 37:911-917 (1959), incorporated herein by reference. The lipid extracts were analyzed for total cholesterol using the assay method of Rudell and Morris, J. Lipid Res., 14:364-366 (1973). Free and esterified cholesterol concentrations were determined following separation by TLC using hexane/ether/acetic acid (70:30:1 (v/v)). Standards were used to identify the area of the plate corresponding to these two lipids, the silica was aspirated and the lipid eluted for assay using chloroform/methanol (2:1 (v/v)). Plasma vesicle phospholipid content was determined by dividing [14C]CHE radioactivity by liposome-specific activity and phospholipid concentrations were determined by the method of Fiske and SubbaRow, J. Biol. Chem., 66:375-400 (1925). Erythrocytes were extracted using the method of Rose and Oklander (J. Lipid Res., 6:428-431 (1965)), followed by a Bligh and Dyer wash to remove residual salts. An aliquot of red blood cells was retained for cell number determination using a Coulter cell counter in order to express cholesterol and phospholipid concentrations as $mmol/10^9$ cells.

Blood was pooled from a group of mice and red cells packed by low-speed centrifugation. The serum was labelled with [3H]cholesterol by incubation for 10 min at 37°C with 100

μCi of radioisotope dried from ethanol. The labelled serum
was added to the packed cells and the mixture incubated at
room temperature for 30 min. The cells were washed and
approximately 10^6 dpm of [^{3}H]cholesterol-labelled cells

5 injected into the experimental groups via the tail vein.
Approximately 1 min after the injection of cells, saline or
liposomes were administered.

Donor and acceptor liposomes were separated
employing ion exchange chromatography. A 10-fold excess of

10 donor vesicles (100 nm diameter) composed of EPC/EPG/Chol
(40:15:45 molar ratio) were incubated with 100-nm or 400-mm
EPC acceptors. Donor liposomes were labelled with
[^{3}H]cholesterol at 5 μCi/100 mg total lipid and acceptors were
labelled with [^{14}C]CHE at 0.5 μCi/100 mg lipid. At specified

15 time intervals, 50 μl aliquots of the incubation mixture (1 mg
acceptor + 10 mg donor/ml) were removed and passed down a
DEAE-Sepharose 6B-CL column prepared in a 1-ml tuberculin
syringe equilibrated with 30 mM NaCl, 20 mM Hepes (pH 8.0).
Columns were spun at 1000 x g for 1 min prior to applying

20 aliquots of the incubation mixture. The liposome mixture was
spun through the column and the eluant (acceptors) obtained
with two subsequent wash/spin cycles with 500-ml aliquots of
buffer. Recovery of ^{14}C-labelled vesicles (acceptors) was
typically >90%. Control experiments in which donors were

25 labelled with a non-exchangeable marker indicated that all of
the donor vesicles bound to the ion exchange column under the
conditions of the experiment. Cholesterol accumulation by
acceptors was determined using an LS 3801 Beckman
scintillation counter equipped with a ^{14}C/^{3}H dual-label

30 program.

Two groups of mice (n=4) were maintained in
metabolic cages and faeces collected daily. After 3 days one
group was injected with 200 μl of saline and the second group
with approx. 6 mg of EPC LUV$_{100}$ (dose 300 mg/kg). Faecal

35 material was collected for a further 7 days. Samples were
extracted using an isopropanol/chloroform extraction procedure
and subsequently assayed for total cholesterol, free
cholesterol and cholesteryl esters, as described above.

Experiments were carried out on mice maintained on regular, laboratory food for rodents (cholesterol excretion rate 10-12 μmol/g faeces) and on Teklad low cholesterol (casein-based diet which resulted in an excretion rate of

5   approx. 0.8 μmol cholesterol/g faeces).

Fig. 1 demonstrates cholesterol mobilization by a homogeneous population of LUV with a mean diameter of 125 nm as determined by QELS (referred to as LUV_{100} and prepared by extrusion as described above). A dramatic increase in plasma

10   cholesterol was observed for animals receiving liposomes (Fig. 1A). Sterol levels peaked 4-8 h after injection at a concentration nearly double that measured in the control mice injected with an equivalent volume of saline. Plasma cholesterol concentrations gradually returned to normal levels

15   after 48 h correlating well with the liposome clearance profile shown in Fig. 1B. Liposomes were labelled with trace amounts of [^{14}C]CHE, a non-exchangeable, non-metabolizable marker frequently used to monitor liposome clearance and distribution in vivo.

20   Using gel filtration as described above, mouse plasma was fractionated and the cholesterol profile determined using the chemical assay procedure of Rudel and Morris. Plasma from control and liposome-treated animals were compared and the results are shown in Figs. 2A and 2B. Fig. 2A shows a

25   normal cholesterol distribution with the majority of cholesterol associated with combined LDL and HDL peaks (fractions 22-50). The elution volumes of VLDL, LDL and HDL were determined as described above. A minor quantity of sterol was detected in the void volume, corresponding to the

30   larger chylomicron and VLDL lipoprotein particles, but quantitatively these fractions represent <5% of the total cholesterol content of the plasma. The elution profile of plasma from liposome-treated animals (4 h time point) is shown in Fig. 2B. The [^{14}C]CHE liposome marker was almost

35   exclusively detected in the void volume, indicating that the LUV_{100} were well separated from the fractions containing LDL and HDL (liposomes smaller than 100-nm diameter are included in the gel and cannot be separated from LDL). The absence of

radioactivity in the remaining fractions indicated that little, if any, assimilation of vesicles into the lipoprotein pool occurred. However, it is possible that small quantities of vesicles had undergone structural transitions to

5 lipoprotein-like particles, but were removed rapidly from the circulation and therefore, not detected. (in vivo)

The cholesterol content of column fractions, shown in Fig. 2B clearly shows that the excess sterol in the plasma of treated mice is associated with LUV. The slight frame shift

10 of peaks between Fig. 2A and Fig. 2B is the result of differences in elution rate and not due to changes in lipoprotein size. Using TLC analysis it was determined that >90% of the liposomal cholesterol was free cholesterol, the remainder being cholesterol ester.

15 The excellent separation of LUV_{100} from the quantitatively most abundant lipoproteins enabled straight-forward isolation and subsequent analysis of the vesicle lipids. Liposome cholesterol accumulation was shown by the increasing C/P ratio of vesicles over a 24-h time-course $\underline{in}$

20 $\underline{vivo}$, as shown in Fig. 3. Consequently, after 24 h the liposomes remaining in the circulation (approx. 10-15% of the initial dose) were in equilibrium with respect to cholesterol and net sterol movement was negligible.

Plasma cholesterol concentrations were measured over

25 a 48-h period in animals treated with a variety of liposomal preparations varying in diameter from 30-250 nm. Sonicated vesicles were prepared as described above. The remaining vesicles were produced by extrusion of MLV through filters with defined pore-sizes to give vesicle populations with the

30 mean diameters described above. Vesicles are referred to by the filter pore size used for their synthesis.

The amount of cholesterol accumulated and removed by liposomes $\underline{in}$ $\underline{vivo}$ is a function of both the rate of cholesterol uptake and the rate of liposome clearance. An

35 estimate of the mass of cholesterol removed from the circulation (mostly by the RES) can be made by calculating the C/P ratio of vesicles $\underline{in}$ $\underline{vivo}$ from plasma concentration of vesicle phospholipid and cholesterol as the excess plasma

concentration above the control at the various experimental time points. All cholesterol above control levels is associated with circulating liposomes. The plasma volume of mice used in these studies was approx. 1 ml, consequently the

5   total amount of phospholipid cleared from the circulation between time points was known. Using the average C/P ratio measured for vesicles between each assay interval an estimate of the amount of cholesterol removed was obtained. The analysis was not continued beyond the point where less than 5%

10   of the initial phospholipid dose remained in the circulation as below this level the measurement error was too large to determine accurate C/P ratios. Fig. 4A shows the cumulative level of cholesterol removed by LUV_{100} up to the time when approx. 5% of the dose remains. After 40 h 2800 nmol of

15   cholesterol were removed from the circulation by the RES, which represents 33 mol% of the injected phospholipid dose. This analysis was used to compare the various liposomal preparations tested. For each preparation the plasma cholesterol and phospholipid clearance profiles were

20   determined and analyzed as described above. The results in Fig. 4B show that LUV mobilize cholesterol most efficiently.

The transfer of sterol from donor vesicles to unilamellar and multilamellar vesicles was studied. Using freeze-fracture electron microscopy and NMR analysis, it has

25   been shown that MLV sized through 400-nm pores retain a number of internal lamellae and therefore cannot be classified as LUV. The transbilayer movement (flip-flop) of cholesterol is rapid, on the order of seconds to minutes in a liquid crystalline bilayer under conditions that promote net sterol

30   flux. Consequently, it was expected that multilamellar systems would act as a good sink for cholesterol as sterol should rapidly disperse through the internal lamellae.

Using an in vitro model in which LUV_{100} or MLV_{400} were incubated with a 10-fold excess of donor liposomes

35   containing tritiated cholesterol as described above, the net transfer of sterol from donor to acceptor was monitored. The rate of cholesterol accumulation in the unilamellar preparation was greater than that observed for the

23

oligolamellar vesicles. It is interesting to note that in the
presence of a 10-fold excess of donor vesicles the equilibrium
C/P ratio of the acceptor should be approx. 0.9:1. The data
in Fig. 5 show that the 100-nm acceptors only achieve a ratio
of 0.35:1 after 8 h at 37°C. This is approximately half the
rate of accumulation observed for the same vesicles in vivo
(Fig. 3).

The cholesterol mobilizing properties of two types
of LUV_{100} were compared. The two types of LUV_{100} were composed
of EPC/EPG (95:5 mol ratio) which is liquid-crystalline at
37°C and DSPC/DSPG (95:5) a gel-state lipid matrix at the body
temperature of the mouse. Phosphatidylglycerol (PG) was
incorporated to impart a surface negative charge, necessary to
prevent the gel-state vesicles from aggregating in the absence
of cholesterol as described in Nayer et al., Biochim. Biophys.
Acta, 986:200-206 (1989), incorporated herein by reference.
Reliable comparison of the two systems was facilitated by
adding a negative charge to the EPC vesicles. The results,
presented in Fig. 6A, reveal that the gel-state vesicles
produced a delayed increase in plasma cholesterol which did
not peak until after 24 h, whereas EPC/EPG vesicles gave rise
to a cholesterol profile similar to that observed for EPC
alone.

The data in Fig. 6A demonstrate that the rate of
cholesterol accumulation for these two types of vesicle was
the same. The different plasma cholesterol profiles occurred
because approximately 70% of the DSPC/DSPG vesicles were
cleared within 4 h compared to less than 30% of the EPC/EPG
LUV_{100} (Fig. 6B). The bulk of cholesterol mobilization
occurred in the first 24 h, consequently liquid crystalline
EPC/EPG removed more than 3000 nmol to the RES, whereas
DSPC/DSPG vesicles removed 1700 nmol. The source of the
accumulated liposomal cholesterol and its fate was determined.
Ultimately, cholesterol efflux must occur from atherosclerotic
plaque to achieve regression. However, it is known that the
cholesterol within cells and atherosclerotic lesions
equilibrates more slowly than sterol present in plasma
membranes directly exposed to acceptor particles. Movement of

this cholesterol will be a secondary event initiated by the primary efflux of outermembrane cholesterol.

In a 20-g mouse approximately 35% of the circulating sterol is associated with lipoproteins and about 65% with the plasma membranes of erythrocytes. However, all of the sterol associated with erythrocytes is free cholesterol, whereas a large proportion of lipoprotein sterol is esterified. Consequently, the largest pool of free cholesterol in the circulation is in the red blood cell plasma membrane. It was found that this source of cholesterol does not change significantly in the presence of liposomes, despite a two fold increase in plasma sterol concentration. This result is shown in Fig. 7A.

Erythrocyte membrane cholesterol can be depleted by liposomes _in_ _vitro_. Consequently it was determined whether erythrocytes act as the primary sterol donor and then rapidly replenished by lipoproteins which are in turn able to extravasate and scavenge more sterol from peripheral tissues. Erythrocytes were isolated from mice and labelled with [^{3}H]cholesterol _in_ _vitro_. The labelled cells were injected into a group of mice, half of which were subsequently treated with saline and half with 300 mg/kg of EPC LUV$_{100}$. The specific activity of red blood cell cholesterol was determined over an 8-h time-course and the two groups compared. As demonstrated in Fig. 7B, the decrease in cholesterol specific activity is the same for both the control and experimental group. Interpretation of these data is limited by the fact that cells labelled _in_ _vitro_ are also removed from the circulation over a similar time-course (determined by chromium labelling). However, it can be estimated that at least 50% efflux of cell sterol would be necessary to account for the rise in plasma cholesterol observed after 8 h. This would result in a considerable dilution of erythrocyte cholesterol if this sterol pool were continuously replenished. As this has not been observed, the data suggest that red blood cell cholesterol is not the primary source of the liposomal sterol accumulated _in_ _vivo_.

C/P ratios of lipoproteins showed a significant
decrease over control values in the first 8 h (Fig. 7C). The
ratio returned to normal values after 8 h mirroring the time-
course of cholesterol accumulation by vesicles. This suggests

5   that it is primarily lipoprotein cholesterol in equilibrium
with circulating liposomes, and that lipoproteins mediate the
transfer of cholesterol from peripheral tissues to liposomes.
The results are also consistent with observations _in vitro_
that indicate cholesterol can undergo desorption from

10 lipoproteins more readily than from erythrocytes. Finally,
the rate of cholesterol accumulation by LUV_{100} _in vivo_ (Fig.
3) is considerably faster than that observed _in vitro_ (Fig.
5), indicating that the rate of cholesterol desorption from
sources _in vivo_ is greater than from the 100 nm vesicle donors

15 used to obtain the data in Fig. 5.

Example 2 -- Regression of Atheromas in Rabbits Treated with
Liposomes

This example demonstrates mobilization of

20 cholesterol and regression of atheromas in rabbits treated
with liposome compositions of the present invention. Plasma
cholesterol concentration increased 2.5 times in liposome
treated rabbits. Aortic lipid content decreased 25% in
liposome treated animals.

25 Egg phosphatidylcholine (EPC) was supplied by
Princeton Lipids (Princeton, NJ). A 0.5% cholesterol
supplemented diet was obtained from Teklad Premier. Blood
collection tubes and butterfly needles (23 gauge) were from
Becton-Dickinson (Missisauga, Ontario). Ketamine, xylazine,

30 heparin, Innovar and Euthanyl were supplied by MTC
Pharmaceuticals, Janssen Pharmaceutics and Organon Technika
(Ontario). Bio-Gel A-15m was purchased from Bio-Rad.
Prepacked Solid Phase silica gel columns were acquired from
Burdick & Jackson. All chemical and solvents were of

35 analytical grade from BDH Chemicals (Vancouver, B.C.)
Forty eight New Zealand White (NZW) rabbits were
housed in wire cages at the Animal Unit of the Research Centre
conforming to guidelines set by the Canadian Council on Animal

Care and the University of British Columbia. The animals were maintained in a controlled temperature environment with a 12 hour dark/light cycle. Approximately 150g of food were given per animal per diem. Water was freely given.

5   Lesions induced in rabbits as a result of maintaining the animals on cholesterol enriched diets for more than two months, do not regress for lengths of up to two years even when they are returned to standard rabbit chow. St. Clair, Prog. Cardiovasc. Dis., 26:109-132 (1983). Even after

10 cessation of cholesterol enriched diets, lesions have been noted to progress and increase in complexity. Prior et al., Arch. Path., 71:82-94 (1961). Moreover, in cases where intermittent feeding schedules were administered or a low cholesterol-enriched diet was given over a period of years,

15 lesions similar to the calcified ulcerated lesions observed in humans have been produced. Constantinides et al., Arch. Pathol., 70:81-92 (1961).

          The correlation between hypercholesterolemia and the onset and progression of atherosclerosis in the rabbit is well

20 established. St. Clair, supra. To ensure that an equal distribution of animals were divided into the respective treatment groups, careful pairing of the animals was done. Initially, the 48 NZW weanlings were screened for responders to the 0.5% cholesterol enriched diet (Teklad diet 0533). The

25 animals were fed the cholesterol diet for one week and plasma cholesterol concentrations monitored until returning to normal. Animals were matched by the extent of the rise in plasma cholesterol levels as well as the rate at which the levels returned to normal. This enabled an equal distribution

30 of animals to be placed into two groups of 24 that were fed either standard rabbit chow or 0.5% cholesterol enriched rabbit chow for 20 weeks to induce atherosclerotic plaque formation. During this time, plasma lipid levels were monitored on a monthly basis. Two animals were euthanized due

35 to complications probably associated with handling and were excluded from the final analyses. After the diet induction period, five animals from each group were sacrificed to verify the formation of lesions and serve as the standards against

which the effectiveness of liposomal treatment was assessed. Thereafter, all remaining animals were fed regular rabbit chow until the conclusion of the study.

Rabbits were fed a 0.5% cholesterol-enriched diet for 20 weeks in order to induce intermediate lesions more significant than fatty streaks associated with shorter duration cholesterol-enriched diets. Chemical and histological analyses of aortas obtained from rabbits following the diet induction period, but prior to treatment, revealed plaques formed that were rich in lipid and surrounded by fibrous tissue. These plaques consisted of almost equivalent amounts of cholesterol and cholesterol ester. The aortic phospholipid in these animals was 15 ± 4 μmol/g wet tissue and aortic total cholesterol was 114 ± 28 μmol/g wet tissue (61 ± 13 μmol/g cholesterol and 53 ± 15 μmol/g cholesterol ester). Animals maintained on a standard diet had aortic phospholipid levels of 4 ± 0.3 μmol/g wet tissue and aortic total cholesterol levels of 10 ± 1 μmol/g which was predominantly cholesterol. The degree of surface plaque involvement in cholesterol fed animals was $78\pm14\%$.

Based on the pairing of plasma cholesterol concentrations, 18 rabbits remaining from each diet group were separated into groups of 9 and were treated with EPC LUV_{100} at a dose of 300 mg/kg or the equivalent volume of saline. Treatment was initiated 4 weeks after return to standard rabbit chow and was given over a 100 day period. The treatment consisted of ten bolus injections of phospholipid or saline administered into the marginal ear vein. One injection was given every 10 days.

The rabbits ranged from 4-6 kg in weight. Each treatment of the vesicle-receiving rabbits required the preparation of approximately 150 mls of LUV_{100} at a concentration of 200 mg/ml. Typically, 6 gram aliquots of EPC were hydrated with 30 ml of filtered 150 mM NaCl, 20 mM HEPES (HBS), pH 7.4, in sterile 50 ml conical tubes, vortexed and kept overnight . As described in Example 1 above,, the resulting multilamellar vesicles (MLVs) were used to generate LUV_{100} by extrusion through two stacked polycarbonate filters

of 100 nm pore size using a 10 ml water-jacketed thermobarrel
Extruder (Lipex Biomembranes, Vancouver, B.C.), according to
the method of Hope et al., Biochim Biophys. Acta, 812:55-65
(1985), incorporated herein by reference. Vesicle sizes were
5   determined by quasi-electric light scattering (QELS) analyses
utilizing a Nicomp Model 370 submicron laser particle sizer
(Pacific Scientific, MD). The vesicles used for the 10
treatments had an average diameter of 114 ± 7 nm.

     A small dose of Innovar™ was given to promote
10   calmness and vessel dilation in animals to ease routine
bleedings necessary for plasma lipid analyses. To facilitate
the final blood collections, ketamine (40 mg/kg) and xylazine
(8 mg/kg) were given intramuscularly to sedate the animals.
Fifty units of heparin (Hepalean) followed by a lethal dose of
15   phenobarbital (Euthanyl) were then perfused into the marginal
ear vein before laparotomy. Organs were removed, rinsed in
saline and immediately frozen in liquid nitrogen. The heart
and full length aorta were collected in one section and kept
in iced saline. The animals were sacrificed in groups of 8-10
20   on alternate days. The organs were randomized prior to
processing and analyses.

     Each aorta was separated from the heart at the
aortic valve and was carefully cleaned to remove any adherent
adventitial fat. The aortas were cut along the ventral
25   surface, opened, and photographed on a black background. The
photographs were used in conjunction with the negatives to aid
in the collection of digitization data as well as to
facilitate the division of the aortas into three regions: the
arch, thoracic, and abdominal aortic segments as described by
30   Rosenfeld et al., Atherosclerosis, 8:338-347 (1988),
incorporated herein by reference. Nine animals were in each
of the 4 treatment groups: (1) vesicle-treated cholesterol-fed
animals (VC), (2) saline-treated cholesterol-fed animals (SC),
(3) vesicle-treated normal diet animals (VN) and (4)
35   saline-treated normal diet (SN). Six aortas from each group
were allocated for lipid analyses and stored at -20°C until
analysis. The remaining three samples in each group were
fixed in 10% neutral buffered formalin for at least 48 hours

and used for gross staining with Sudan IV and histology.
Holman et al., Lab. Invest., 7:42-47 (1958), incorporated
herein by reference. At the time of lipid analysis, the
aortas were patted dry and divided into the three segments.

5   Wet weight and length were measured and the aortic segments
were homogenized (Polytron) in HBS. Two additional washes of
the Polytron probe with HBS were collected for each segment to
ensure complete homogenate recovery.

Whole aortic segments were analyzed by digitization.

10 In this analysis, photographic negatives obtained from all
unstained aortas were illuminated generating an image using a
Microcomputer Imaging Device (Imaging Systems). The
percentage of plaque involvement was calculated by dividing
the area occupied by surface plaque by the area of the entire

15 aorta segment. Distinct differences were observed in the
degree of shading of plaques and uninvolved aortic tissue.
Assessments of the percentage of atherosclerotic plaque
involvement were performed by two observers and the results
were averaged. Interobserver variation was within ±5%.

20 Cholesterol and phospholipid content of the aortas
and livers of the sacrificed animals were quantified following
Bligh and Dyer extractions of the homogenates. Bligh and
Dyer, Can. J. Biochem. Physiol., 37:911-917 (1959),
incorporated herein by reference. Total cholesterol,

25 cholesterol, and cholesterol ester contents were determined
according to the method of Rudel and Morris, J. Lipid Res.,
14:364-366 (1973), incorporated herein by reference.
Cholesterol and cholesterol esters were separated by silica
gel chromatography on Burdick and Jackson prepacked 200 mg

30 Solid Phase Silica Gel columns. Cholesterol esters were
eluted with 1 ml methylene chloride. Cholesterol was
collected following methylene chloride/methanol (95:5) elution
after transferring the columns to a new carrier. Phospholipid
content was measured according to Fiske and Subbarow, J. Biol.

35 Chem., 66:375-400 (1924), incorporated herein by reference.
Lipoprotein lipid profiles were quantified by enzymatic
procedures after phosphotungstic acid precipitation.

Aliquots of aorta or liver homogenates were incubated overnight at 37°C with 1 ml of 1N NaOH. Thereafter, sodium dodecylsulphate (SDS) was added to the mixture to make a 1% solution needed to solubilize any remaining particulate

5 matter. Protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay method (Pierce Chemical Company, Rockford, IL) after incubation for 1 hour at 60°C and read at A_{562} against an albumin standard.

Typically, 2-3 mm segments from the arch, thoracic,

10 and abdominal aorta of three different animals within each treatment group were divided into left and right halves and embedded in paraffin. At least 8 segments from each region were prepared as blocks, depending on the length of the aorta. Alternate sections of 5 μm were adhered to gelatin coated

15 slides from paraffin blocks and visualized with hematoxylin and eosin (H&E) or Weigart's-van Gieson's stains. Intima/ media ratios of the different regions were calculated by initially measuring an average ratio from 3 photographs generated from each section and using this value to determine

20 a final mean±standard deviation from all the sections made from the animals of each group.

The nature of plaques from animals sacrificed after the diet induction period, but prior to any treatment was examined after sections were made from segments held into

25 place with tissue mount (OCT) on wooden stages and quick frozen in isopentane followed by liquid nitrogen. Subsequently, alternate sections of 5 μm were adhered to polylysine coated slides and visualized with Sudan IV differentiated with Harris' hematoxylin, H&E or van Gieson's

30 stains to highlight lipids and collagen.

Unless otherwise indicated, mean±standard deviation values are presented. The significance of the difference of the means was assessed by an analysis of variance using the two-sample t test. Only values of $P<0.05$ were considered

35 significant.

During the course of this study, animals maintained on the atherosclerotic diet exhibited plasma total cholesterol concentrations ranging from 5-10 times that of the control

animals fed the standard diet while fed the cholesterol-
enriched diet. The cholesterol concentrations remained
elevated (2-5 times higher) until the conclusion of the study
even though standard rabbit chow was given during the
5   treatment period. This is illustrated in a typical time
course of cholesterol mobilization resulting from the infusion
of 300 mg/kg EPC LUV_{100} or an equivalent volume of saline
demonstrated in Fig. 8. A comparison of control animals
injected with saline demonstrates that animals previously fed
10   the high cholesterol diet (panel A) maintained plasma
cholesterol concentrations 3 times higher than animals
maintained on the standard diet throughout the study (panel B)
even though the cholesterol diet was terminated 10 weeks
earlier. Despite the atherosclerotic animals having excess
15   plasma cholesterol, an injection of LUV_{100} resulted in a
dramatic 2.5 times increase in plasma cholesterol
concentrations in both hyper- and normocholesterolemic animals
when compared to saline treated counterparts. Plasma
cholesterol levels peaked at 24 hours post-infusion before
20   returning to baseline levels after 5 days. This time course
correlates with the removal of vesicles from the circulation
measured as total plasma phospholipid concentration
illustrated in the clearance profiles shown in Fig. 9.
Although atherosclerotic animals had slightly higher total
25   phospholipid concentrations, similar clearance kinetics of the
injected vesicles were seen between normal and
hypercholesterolemic rabbits.

    As demonstrated in Example 1 above, the amount of
cholesterol accumulated and removed by liposomes with each
30   infusion is a function of the rate of liposomal cholesterol
uptake and the rate of vesicle clearance. Also, it was
determined that all cholesterol above saline treated levels
was associated with circulating liposomes by generating a
cholesterol and phospholipid profile after separating vesicles
35   from plasma by gel filtration. This showed that excess plasma
cholesterol was associated with the vesicles and that >90% of
the cholesterol was free cholesterol. Hence, an estimate of
the mass of cholesterol removed from the circulation (mostly

by the RES) was made by calculating the C:P ratios of vesicles at intervals following each injection from plasma phospholipid concentrations (vesicle-treated concentration minus saline-treated concentrations) and cholesterol (excess plasma

5   concentration above the control concentration) at different time points during the experiments.

The plasma volume of the rabbits was approximately 150 ml. An estimate of the cholesterol removed was calculated employing the average C:P ratio measured for vesicles at each

10 assay interval. This data is shown in Fig. 10. The data represents an average±standard deviation expressed as mmol of cholesterol removed with each treatment in hypercholesterolemic animals and was calculated from data obtained from treatments 1, 4 and 10. The analysis was not

15 continued beyond the point where less than 10% of the initial phospholipid dose remained in the circulation. Below this level, the measurement error was too large to determine accurate C:P ratios. After 104 hours it was estimated that approximately 1 mmol of cholesterol was removed from the

20 circulation by the RES, which represents approximately 50 mole % of the injected phospholipid dose. Furthermore, based on plasma cholesterol concentrations measured in animals 24 h post-injection, each of the 10 infusions of liposomes caused dramatic cholesterol mobilization.

25   The ability of the animals to tolerate and remove repeated injections of phospholipid and the consequences of administering excess phospholipid on plasma lipid levels were examined. Chronic short term (one week) administration of Intralipid, an emulsion of triglycerides and phospholipids,

30 causes increased LDL levels. Although the phospholipid content of Intralipid is comparable to the dose of 300 mg/kg LUV_{100} per injection of the present treatment regimen, Intralipid is generally given intravenously on a daily basis as a nutritional supplement.

35   Each injection of 300 mg/kg EPC LUV_{100} apparently induces a transient 100-fold increase in plasma phospholipid concentrations and at the end of liposomal therapy (10 injections) each animal received an average total dose of

12-20 mmol (10-15g) of phospholipid. The clearance profiles
of several injections of EPC LUV_{100} in cholesterol fed rabbits
is shown in Fig. 11A. As illustrated, significant differences
in the rates of vesicle clearance between injections were not
5   detected. Fig. 11B shows that similar concentrations of
vesicle phospholipid remain in the circulation 24 h post-
injection in both normo- and hypercholesterolemic animals
following serial injections. If the ability of the fixed
macrophages of the RES were compromised, increasing
10   phospholipid levels would likely be detected during the later
treatments. Furthermore, 5 days post-injection, the injected
dose of liposome phospholipid was completely removed from the
circulation and plasma phospholipid and cholesterol
concentrations returned to baseline levels.
15   At the conclusion of the study, saline-treated
cholesterol-fed animals maintained elevated plasma cholesterol
levels whereas vesicle-treated animals had levels comparable
to animals maintained on the standard diet. The reduction in
plasma cholesterol concentrations of vesicle-treated
20   atherosclerotic animals resulted from a reduction in both
plasma LDL and HDL cholesterol concentrations although the
relative proportions of HDL/LDL cholesterol were not affected.
No changes in the plasma lipid profiles (cholesterol,
phospholipid or triglycerides) were detected in animals
25   maintained on standard rabbit chow throughout the study.
Plasma phospholipid levels in vesicle-treated animals were
similar to their saline-treated counterparts despite the
injection of approximately 15 grams of phosphatidylcholine per
animal during liposomal therapy. These results, unlike those
30   observed with Intralipid infusions, suggest that repeated
administration of LUV_{100} given at 10 day intervals does not
compromise RES function or normal plasma lipid homeostasis.
          Erythrocyte cholesterol remained constant throughout
the infusions. However, a decrease in the C:P ratios of
35   lipoproteins was detected over the first 24 hours. This C:P
reduction gradually returned to normal levels after 48 hours
(see Fig. 12). This time course mirrors cholesterol
accumulation by the vesicles. These results suggest that the

lipoprotein pool of cholesterol rapidly equilibrates with the vesicles and supports the hypothesis that liposomes generate cholesterol-poor lipoprotein particles that can access peripheral tissues and promote cellular cholesterol efflux.

5   The extent of lesion progression or regression was assessed by three complementary methods: (1) chemical lipid and protein assays to determine lesion bulk, (2) digitization of gross surface morphology to quantitate the degree of plaque involvement, and (3) histochemistry to examine the nature and

10   depth of the lesions.

Despite elevated plasma cholesterol concentrations persisting in animals returned to standard rabbit chow, saline-treated animals were found to have arterial wall cholesterol content expressed per gram wet weight of 94 ± 12

15   μmol/g total cholesterol, 58 ± 6 μmol/g free cholesterol and 37 ± 9 μmol/g cholesterol esters with an average surface plaque involvement of $77\pm17\%$. Although there appears to be slight reduction in the cholesterol ester content, the values of the lipid content of saline-treated animals were not significantly

20   different from values found in atherosclerotic animals prior to treatment indicating that there was no progression or regression of lesions after 4 months. On the other hand liposome-treated animals were found to have significantly less cholesterol content of the entire aorta with levels of 85 ± 8

25   μmol/g total cholesterol, 48 ± 5 μmol/g free cholesterol and 37 ± 6 μmol/g cholesterol esters. Because there were no significant differences between the lipid content of animals before or after saline treatment, the reductions in plaque cholesterol content between liposome- and saline-treated

30   animals indicates regression, not simply decreased progression, of plaques.

Aortic lipid content was expressed per gram of protein weight as wet weights are likely to be more variable. No significant differences were found between the protein

35   levels in both saline- and vesicle-treated animals. The protein content of the aortas to be 0.41 g protein/g wet weight and 0.43 g protein/g wet weight, respectively. Expressing the data per g protein, liposomal therapy resulted

in a 25% reduction in total cholesterol content of the entire
aorta of vesicle-treated animals compared to saline-treated
controls. By segment, there was a 48% reduction seen in
thoracic aorta cholesterol levels and small reductions in the

5   arch and abdominal aortas (see Fig. 13A). Significant
reductions in the cholesterol ester levels in vesicle-treated
animals were also noted and again the thoracic aorta
demonstrated the greatest decrease (see Fig. 13B). In
addition to decreased cholesterol content, aortic phospholipid

10 levels in vesicle-treated atherosclerotic animals decreased,
although not to the level of statistical significance.

        In order to maximize the number of animals within
each group, all negatives generated from photographed
unstained aortas were digitized. Gross Sudan IV staining of 3

15 aortas from each treatment group confirmed the same degree of
surface plaque involvement as unstained aortas. The area of
plaque involvement was determined by digitization. The data
is shown in Fig. 13C. Liposome-treated, cholesterol fed
rabbits demonstrated 61±13% involvement of the entire aorta

20 compared to 77±17% involvement of saline-treated animals,
representing an overall 16% reduction of surface plaque. In
agreement with the reductions in cholesterol content detected
by lipid analyses, the thoracic aorta exhibited the most
benefit from liposome infusion with digitization analysis and

25 displayed a 26% reduction in plaque involvement, whereas the
abdominal aorta revealed a 16% reduction. There was a slight
reduction in the degree of surface plaque involvement of the
arch that failed to reach statistical significance. No
significant differences between treated and untreated control

30 animals maintained on the standard diet were seen and both
groups showed essentially no plaque involvement.

        Histochemical analysis revealed extensive raised
plaques (intimal thickening) in the cholesterol fed animals as
expected from gross surface morphology inspection. Whereas

35 digitization quantitated the extent of plaque involvement,
histochemical analyses allows the depth and nature of the
lesions to be assessed. Generally, the plaques exhibited
extensive intimal thickening due to stratified lipid deposits

that were surrounded by a collagenous network. The arch region was noted to display more advanced lesions of apparent crystalline cholesterol deposits and showed a few isolated necrotic foci as detected with H&E staining.

5   Representative sections of the thoracic aorta of vesicle-treated and saline treated animals revealed that lesions of animals treated with vesicles manifested fewer lipid deposits and showed moderately reduced plaque thickening when compared to saline treated atherosclerotic animals. This

10   is quantified in Table 1 summarizing the data obtained from the analysis of pictures taken from multiple sections used to assess the severity of lesions present in the arch, thoracic or abdominal aorta of atherosclerotic animals. As can be seen, a decrease in the intima/medial ratios in the arch and

15   thoracic regions of liposome treated animals were detected, whereas no changes were detected in the abdominal aorta. No apparent differences were detected between treated and untreated animals maintained on the standard diet throughout the study.

20   Cholesterol feeding of rabbits often leads to the accumulation of cholesterol in a number of tissues including the liver. However upon the return to regular rabbit chow, non-arterial tissue cholesterol levels often revert to normal within a month. Liver cholesterol content was measured in

25   order to gain insight into whether (1) increased biliary excretion of cholesterol might be occurring in liposome-treated animals due to massive deposition of the injected phospholipids in the liver resulting in reduced liver cholesterol levels or (2) there was a detrimental accumulation

30   of cholesterol mobilized by the liposomes to the liver. In atherosclerotic animals, liposome-treated rabbits demonstrated a slight reduction in liver cholesterol content having average levels of 8 μmol/g that are comparable to control animals fed the standard diet. Saline-treated animals exhibited average

35   levels of 11 μmol/g. This difference was not statistically significant.

All publications, patents and patent applications mentioned in this specification are herein incorporated by

reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

5   Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Table 1. Measurement of Intimal/Medial ratios in the different regions of the aorta of vesicle and saline treated atherosclerotic animals.

Intimal/Medial Ratios

Portion of aorta	Liposome Treated	Saline Treated	Significance (P value)
Arch	1.51±0.55	1.76±0.94	N.S.
Thoracic	1.34±0.73	1.93±1.12	P< 0.01
Abdominal	1.84±0.95	1.81±1.25	N.S.